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Temperature-Dependent Macrophage Activity in Rainbow Trout

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Temperature-Dependent Macrophage Activity in Rainbow Trout

An Honors Thesis submitted in partial fulfillment of the requirements of Honors Studies in Biological Sciences

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Abstract

Fever is an essential component of the immune response. Fever enhances immune responses as well as creates an environment in which the body has advantages over pathogens. Macrophages are often the first cells that come in contact with pathogens, as they reside in tissues. They are important for their engulfment of pathogens that results in the digestion of the pathogen, but they also produce nitric oxide and cytokines that contribute to immune response in a variety of ways, including initiating adaptive immunity and directing the production and activity of other immune cells. We were curious about macrophages from other temperature environments–including fish. The RTS11 cell line, derived from the spleen of rainbow trout, was utilized here for studies on the influence of fever temperature on its macrophage functions. During exposure to a variety of temperatures, macrophage metabolism and activity was measured using protein synthesis and production of nitric oxide. Even without stimulation, a temperature of 19°C alone can increase nitric oxide production and protein synthesis in RTS11 macrophages. Macrophage activity was stimulated using lipopolysaccharide, poly I:C, and peptidoglycan, mimics of or actual components found in viruses or bacteria known to stimulate macrophage activity in mammals, aves, and fish. Results from these experiments indicate that the effects of stimulations on nitric oxide production and protein synthesis depend on whether the stimulation has viral or bacterial origins, with viral stimulations raising nitric oxide production and protein synthesis consistently and bacterial stimulations causing more variable responses. These results indicate that moving to a warmer part of the stream initiating a behavioral fever temperature has a major effect on the activity of macrophages and therefore on the entire immune response.

Introduction

Some form of fever has been highly conserved by natural selection among cold-blooded and warm-blooded vertebrates for over 600 million years of evolution (Evans, et al). Fever temperatures therefore clearly provide a significant survival advantage to organisms. Fever stimulates both innate and adaptive immune responses and is known to be beneficial for the following reasons: bacterial and viral pathogens replicate less efficiently, adaptive immunity is more potent, and human cells are more resistant to the toxic effects of cytokines at higher temperatures (Parham). Fevers also induce lethargy to conserve energy, allowing the body to invest a greater amount of energy in immune responses (Parham). However, the use of antipyretic therapy, aggressively attempting to regulate febrile temperatures, is commonly used in medicine today despite the known benefits of increased temperature in an immune response (Schulman, et al). In a study conducted in an intensive care unit, patients receiving antipyretic therapy had a higher mortality rate than patients whose febrile temperatures were only treated when they rose above 40°C (Schulman, et al). Clearly it is relevant and very important to the advancement of modern medicine that we better understand the role and methodology of fever.

The coordination of the fever response involves numerous complex pathways that are yet to be fully understood. However, we know that many cell types are involved including dendritic cells, natural killer cells, neutrophils, B and T lymphocytes, vascular endothelial cells, and macrophages (Evans, et al).

Resident macrophages are extremely important in an organism's defense against an invading pathogen as they are frequently the first immune system cells to encounter pathogens due to their residence in tissues throughout the body. In addition to their phagocytosing role in the initial stages of an immune response, macrophages are also responsible for releasing a variety of signaling proteins called cytokines that are important in inducing the adaptive immune response. Specifically, pro-inflammatory cytokines and signaling molecules like nitric oxide (NO) have vital roles in regulation of innate and adaptive immune system responses. These cytokines are tumor necrosis factor-alpha (TNF-a), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-12 (IL-12).

Their functions include enhancing T-cell proliferation, inducing production of acute phase proteins, and elevating core temperature (Kirkley, et al). The expression of signaling molecules and cytokines is known to be temperature-dependent. In research done with a mouse macrophage line, TNF-a and IL-1 were produced at a higher concentration at 31°C when compared to 37°C while IL-6 was produced in higher concentrations at temperatures greater than 37°C (Kirkley, et al). The signaling molecule nitric oxide is used in this research to quantify macrophage activity.

Nitric oxide is known to have direct antimicrobial properties against many pathogens including bacteria, yeasts, protozoa, helminths, and viruses (De Groote and Fang). Numerous studies, mostly conducted using mice macrophages, suggest that nitric oxide is free to diffuse across membranes and therefore has both intracellular and extracellular antimicrobial effects (De Groote and Fang). Recent research has shown that nitric oxide itself causes a second increase in glycolysis in mouse macrophages (Kam, et al). To get enough nitric oxide the combination of a bacterial signal (lipopolysaccharide) as well as a T-cell cytokine IFN-g must be given (Kam, et al). Thus, the product of the enzyme inducible nitric oxide synthase (iNOS) increases rates of glycolysis (Li, et al). This enhances the immune response by giving macrophages more energy to fight off infections.

While similar cytokines and signaling molecules are used, ectotherms (cold-blooded vertebrates) employ a different method of fever regulation than endotherms. Ectotherms including reptiles, fish, and insects raise their core temperature during infection through behavioral regulation, in which animals seek warmer environments when they are ill despite the risk of predation (Covert and Reynolds).

Behavioral regulation has been shown to be very effective in ectothermic immune responses. Previous studies of desert iguanas showed that survival was reduced by 75% when the iguana was prevented from seeking warmer environments (Evans, et al). Interestingly, antipyretic drugs prevented the desert iguana from participating in this behavior (Evans, et al). Therefore, it is likely that similar biochemical pathways are involved in fever production and regulation in endotherms and ectotherms.

Fish have not yet been extensively tested, but it is known that fish like many other ectotherms choose to go to warmer areas of their environments when they have infections

(Covert and Reynolds). Initial research with fish showed that fish that maintained a higher febrile temperature had a higher survival rate when injected with a pathogen (Reynolds, et al). A study of sockeye salmon demonstrated that higher temperatures do not kill the virus, but instead enhance the immune response of the salmon (Covert and Reynolds). Therefore, we were curious about the direct effects that temperature has on the immune response in fish. Previous research defines a complex cytokine and chemokine network in fish that functions to regulate physiological and immunological processes, making rainbow trout a suitable model organism (Simon, et al). The RTS11 cell line used in this research was derived from rainbow trout spleen. RTS11 cells are predominantly non-adherent, but include a population of mature, adherent macrophage-like cells. The non-adherent cells are likely a precursor cell at an earlier stage of macrophage development (Ganassin, et al). Therefore, this cell line can be used to study the effect of fever temperature on macrophage activity.

Materials and Methods

Routine RTS11 Cell Culture

RTS11 cells were continuously cultured in the lab. They were kept in an incubator set to the normal body temperature of rainbow trout, 16°C. RTS11 cells were routinely cultured in flasks with HyClone Leibovitz L-15 media. Color change of the L-15 media from the original red tint to a yellow tint signified the need to replace the media, typically within three to four weeks of initial culture.

Cells were typically cultured with 30% fetal bovine serum (FBS) as a high concentration was needed to maintain proliferation. This is likely due to essential growth factors found in FBS (Ganassin, et al). However, response to LPS was found to be greatly impacted by the presence of FBS. Therefore, media containing 0.1% FBS was used when experimenting with RTS11 macrophages.

Cell Count

In preparation for an experiment measuring the nitric oxide production or protein synthesis of RTS11 macrophages, cells were gathered from culture flasks by scrubbing adherent cells from flasks with a Cell scraper by VWR for large flasks and a Cell scraper by Falcon for small flasks. Cells were then transferred to 50 mL tubes for centrifugation at 1000 rpm for 2 minutes. The supernatant was removed and discarded. The pellet was mixed with 1 mL of L-15 media. Based on a visual estimate of the size of the pellet resulting from the centrifugation, a dilution was created using 10 ul of the cell solution with the appropriate amount of media in order to create the desired solution aiming for a concentration of $1x10^4$ to $1x10^7$ for accurate counting. This new solution was vortexed.

10 ul of the diluted cell solution was combined with 10 ul trypan blue. This was mixed and placed in a chamber that was inserted into the Countess Automated Cell Counter from Invitrogen. The cell count included numbers of total, live, and dead cells, and a percentage of viability. The number of live cells was used to design experiments.

Stimulation of Macrophages

Lipopolysaccharide (LPS) is a component of the cell wall of gram-negative bacteria, and therefore can induce responses similar to that of bacterial infections in macrophages. LPS is a common stimulant used in immunological research and has been used extensively in the Durdik lab. It was used at concentrations of 10 ug/ml in nitric oxide assay experiments and protein synthesis experiments.

Peptidoglycan (PGN) is a component of the cell wall of both gram-positive and gram-negative bacteria. It therefore also induces a response to a bacterial infection. It was used in a concentration of 1 ug/ml and 5 ug/ml in nitric oxide assay experiments and at concentrations of 5 ug/ml and 10 ug/ml in protein synthesis experiments.

Polyinosinic:polycytidylic (poly I:C) is a synthetic double-stranded RNA analog (Harris, et al). It is a molecular pattern associated with viral infections, acting as a potent viral mimic agent, interferon inducer, and antitumor agent (Teng, et al). Therefore, it can stimulate responses in RTS11 macrophages similar to a viral infection. Poly I:C was chosen as a stimulant because it has been previously shown to induce innate and adaptive immune responses specifically in fish species (Lulijwa, et al). It was used at concentrations of 5 ug/ml and 50 ug/ml in nitric oxide assay experiments and at 50 ug/ml in protein synthesis experiments.

Nitric Oxide Griess Assay

To conduct an experiment measuring the nitric oxide production of macrophages, L-15 media without phenol red was added to the macrophages to create a solution with the desired number of cells, either 100,000, 200,000, or 500,000, per 100 ul. Media without phenol red was necessary so that it would not interfere with measurements of wavelength, which is how nitric oxide levels are quantified. After this dilution was created, the cells were kept on ice while the stimulants were prepared.

96 well plates were designed so that triplicate repeats were made of each treatment condition, shown in Figure 1. One plate was created for incubation at each desired temperature. 100 ul of the cell solution was added to each well. 100 ul of stimulant solutions were distributed into each desired well, either immediately after cell placement or after a 2-hour incubation period to allow cells to adhere.

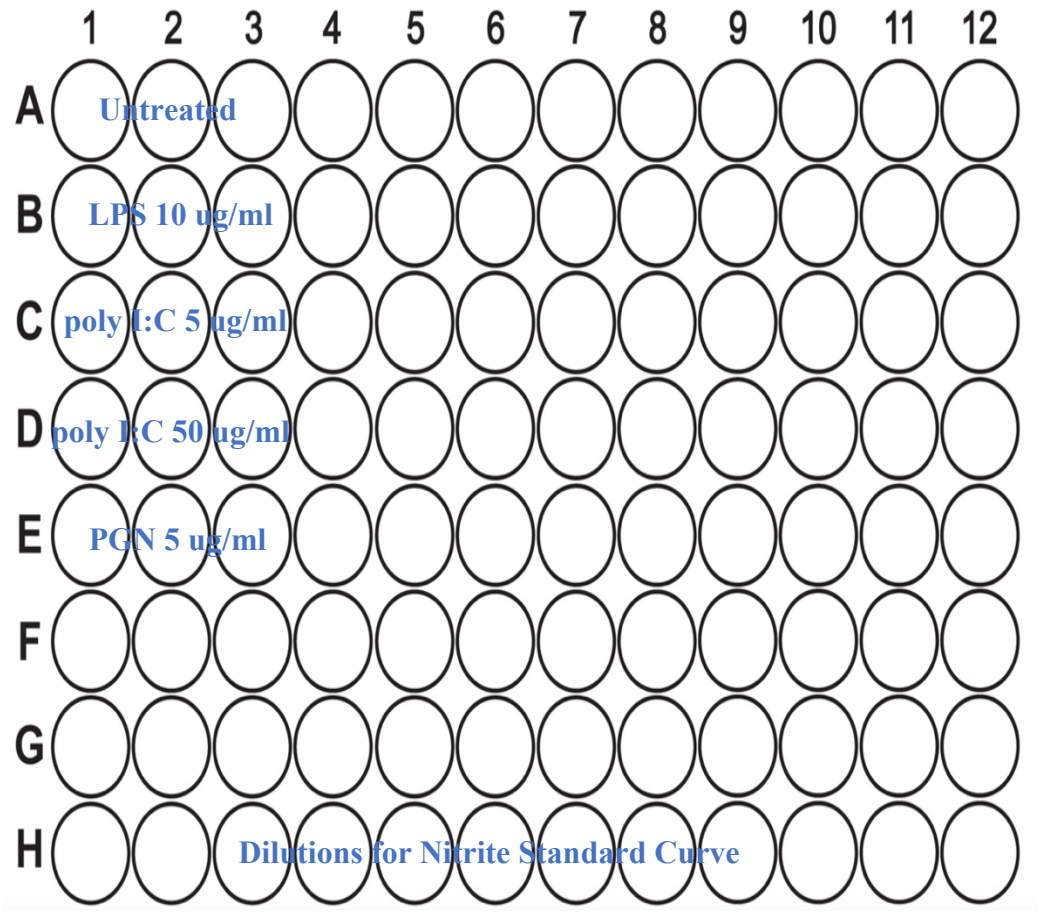


Figure 1. 96-well plate design for NO experiment.

Macrophages were incubated at various temperatures in order to analyze the different amounts of nitric oxide production at different temperatures. 16°C, the normal body temperature of rainbow trout, was always used. 19°C was also always used, as this was found during initial experiments in the Durdik lab to be a successful fever temperature for fish. 18°C, 20°C, and 21°C were used more variably to analyze a wider range of fever temperatures. Macrophages were incubated for various time periods at these temperatures, including 24 hours, 48 hours, 72 hours, and 96 hours.

The Griess reagent system measures nitrite (NO_2^-), a stable and nonvolatile product of nitric oxide (NO). A chemical reaction involving two reagents–sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED)–is used to detect nitrite concentration via

a plate reader at a wavelength of 570 nm. The chemical structure and reaction of these reagents is shown in figure 2.

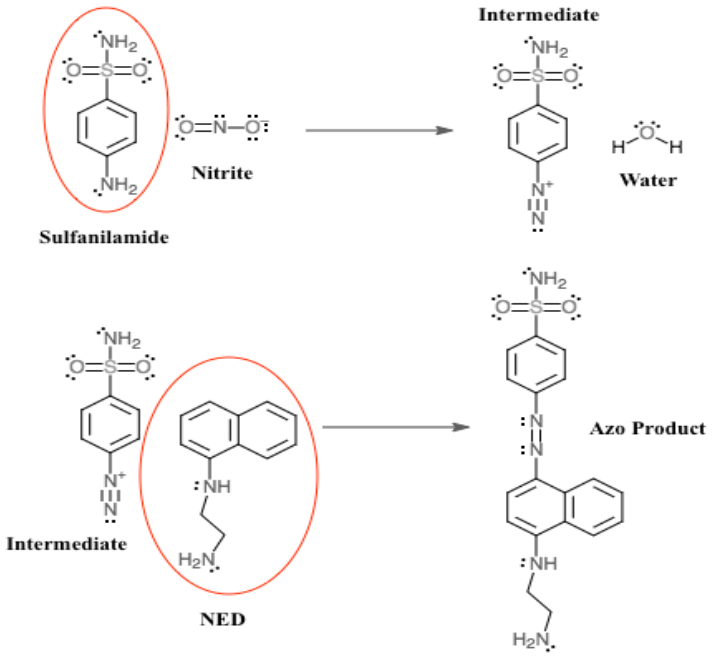


Figure 2. Chemical reaction occurring in the Griess reagent system, creating an azo compound that can be measured via a plate reader at a wavelength of 570 nm.

Prior to removing cells from incubation, 100 ul of standard diluted nitrite solutions of 1M $NaNO_3$ and H_2O were prepared in the bottom row of a 96 well plate, also shown in figure 1. The standard contained concentrations of nitrite at 0, 0.3, 0.7, 1, 3, 6, 10, 20, 40, 90, and 190 um. This created a Nitrite Standard reference curve used to calculate the amount of nitrite produced as a byproduct of the NO produced by macrophages.

After removal from various incubation locations, 100 ul of supernatant from cells was removed and placed in the new 96 well plate containing the Nitrite Standard. Sulfanilamide and NED reagents were also equilibrated to room temperature from their preferred storage temperature of 4°C. Using a multichannel pipettor, 50 ul of sulfanilamide was distributed to each well containing experimental supernatant and each well in the dilution series for the nitrite standard reference curve. After a ten-minute incubation period in a place protected from light, a multichannel pipettor was again used to distribute 50 ul

of NED solution to all wells. Another ten-minute incubation period in a place protected from light was required before measuring the absorbance of the formed azo compound at a wavelength of 570 nm using a Biotech plate reader. Data was then analyzed via Excel.

Click-iT HPG Alexa Fluor Protein Synthesis Assay

The Click-iT HPG alexa flour protein synthesis assay was used to measure levels of protein synthesis. Click-iT HPG (L-homopropargylglycine) is an amino acid analog of methionine that contains an alkyne moiety. A chemoselective ligation reaction between an azide and an alkyne takes place upon incorporation into proteins, and the alkyne-modified protein can be detected by alexa fluor 488.

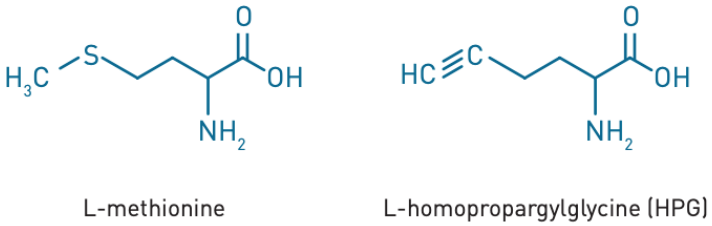


Figure 3. Structures of L-methionine and L-homopropargylglycine (Click-iT HPG).

To conduct an experiment measuring the global protein synthesis of macrophages, L-15 media was added to the macrophages so that there were 1 million cells per 500 ul. After this dilution was created, the cells were kept on ice while the stimulants were prepared.

L-15 media without methionine was used to create stimulation solutions. Click-iT HPG was added in the stimulant solutions at a concentration of 1 ul per 1 ml. 24-well plates were designed so that triplicate repeats of each stimulant condition were created, shown in Figure 4. One plate was created for incubation at each desired temperature.

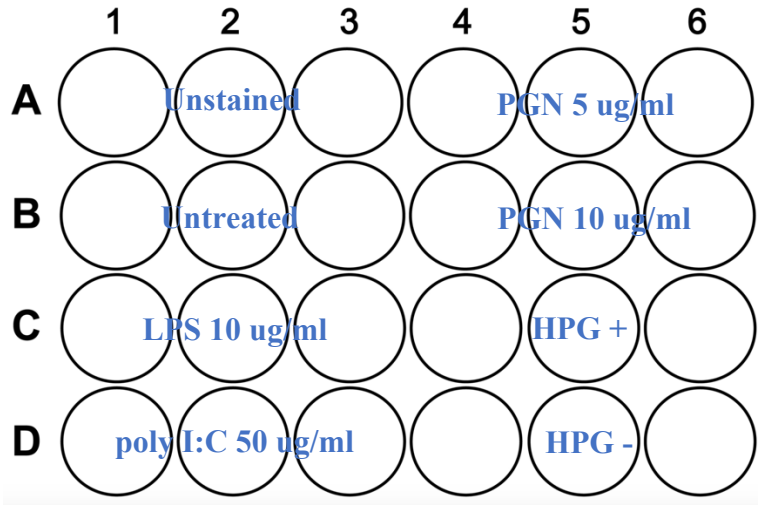


Figure 4. 24-well design for protein synthesis experiment.

500 ul of the previously made cell solution containing normal media with methionine was placed in six wells to serve as controls. 500 ul media was added to create equal amounts in these wells. The remaining cell solution was centrifuged, and the supernatant was removed. An equal amount of media without methionine was then added to the cells to create a solution of 1 million macrophages per 500 ul in media without methionine. 500 ul of the new cell solution was then added to the remaining desired wells. 500 ul of each stimulation treatment which contained Click-iT HPG was distributed to the desired wells.

Positive HPG (HPG+) and negative HPG (HPG-) triplicates were also created to set parameters (maximums and minimums) on the flow cytometer. 500 ul of cell solution containing media without methionine was distributed to three wells, and 500 ul of any leftover treatment solutions which contained Click-iT HPG were added to serve as the HPG+ control. 500 ul of cell solution containing media without methionine was distributed to three wells, and 500 ul of media without methionine was added to serve as the HPG- control. Only one set of triplicates was created for each HPG+ and HPG-, and these were created on the plate incubated at normal body temperature, 16°C.

Plates were incubated for four hours at 4°C, 16°C, 19°C, and 20°C. After incubation, cells were removed from plates and placed into 1.7 ml tubes. The cells were washed once with 100 ul phosphate-buffered saline (PBS). Then, 100 ul of a solution of

3.7% formaldehyde in PBS was added to cells, serving as a fixative. After rest in room temperature for fifteen minutes, cells could be stored for up to one week at 4°C.

After fixation, cells were washed twice with 100 ul of 3% bovine serum albumin (BSA) in PBS. Then, 100 ul of 0.5% Triton in PBS, a cell permeabilization buffer solution, was added to each tube. Cells were incubated with this solution at room temperature for 20 minutes.

During this time, the reaction cocktail that would allow fluorescence to be measured was created. The components of the cocktail were added according to kit instructions in the amounts as shown in Table 1.

Reaction Components	Amount needed (for 2 plates or 25 coverslips)
1X Click-iT® HPG reaction buffer (prepared in step 1.4)	21.5 mL
Copper (II) Sulfate ($CuSO_4$) (Component D)	1.0 mL
Alexa Fluor® azide (Component B)	62.5 µL
1X Click-iT® HPG buffer additive (prepared in step 5.1)	2.5 mL
Total volume	25 mL

Table 1. Reaction components for the Click-iT HPG alexa fluor protein synthesis assay. Calculations were done in order to make the required amount of cocktail for the number of samples in the experiment.

After the 20-minute incubation period, cells were washed twice with 100 ul 3% BSA in PBS. Then, 100 ul of the cocktail was added to each sample. The solutions were mixed thoroughly, and the cells incubated for 45 minutes in a location protected from light.

The samples were then washed with 100 ul of the Click-iT reaction rinse buffer (Component F). Finally, 100 ul of staining buffer was added to each sample. Fluorescence was measured using a flow cytometer. The online software FlowJo (version 10.7.1) was used to analyze data.

Results

Nitric Oxide Assay

Figure 5 depicts the results of a typical nitric oxide assay experiment. This experiment compares the nitric oxide production at normal body temperature, 16°C, versus fever temperatures of 19°C and 21°C. In the untreated condition, a fever temperature of 19°C alone caused a significant increase in nitric oxide production. This experiment also shows an increase in nitric oxide production at the increased fever temperature of 21°C in the untreated condition.

In each of the stimulation conditions in this experiment, nitric oxide production remained highest at 19°C. Poly I:C at a concentration of 50 ug/ml appeared to cause an increase in nitric oxide production at 16°C, and a slight, highly variable increase at 19°C beyond the increase at 19°C with no treatment (without poly I:C). The rest of the stimulation conditions produced nitric oxide concentrations that did not vary significantly from the untreated condition.

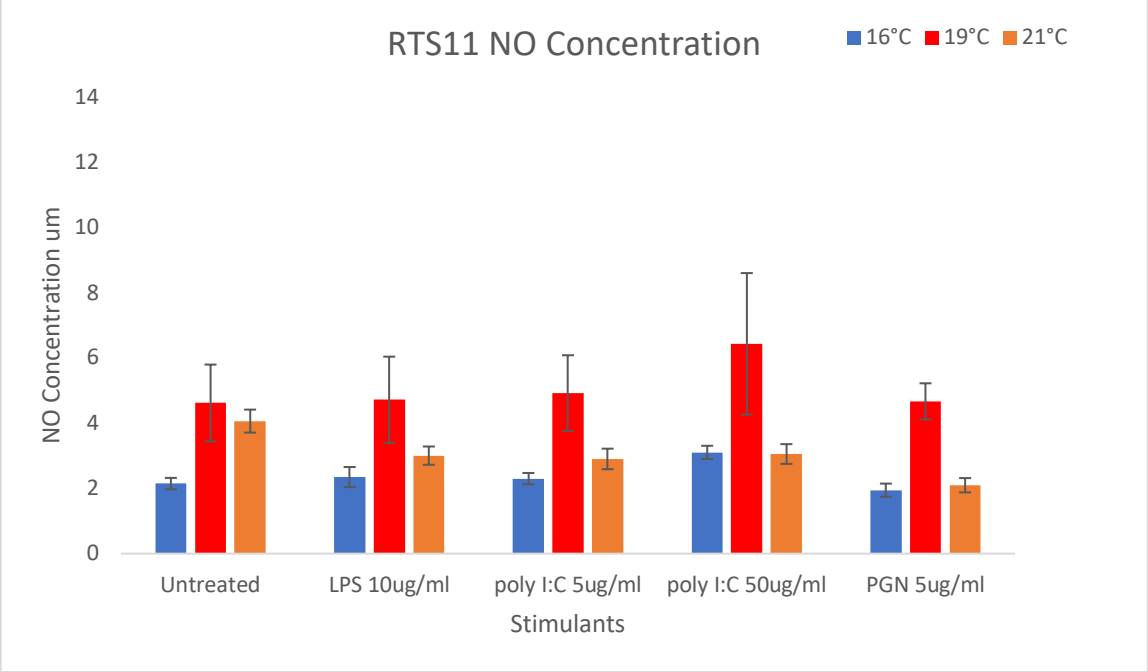


Figure 5. Impact of fever temperature and stimulation on nitric oxide production by RTS11 macrophages. Cells were incubated at 16°C, 19°, or 21°C for 48 hours. 100,00 cells were

placed in each well. Cells were either stimulated immediately after plating with 10 ug/ml LPS, 5 or 50 ug/ml poly I:C, or 5 ug/ml PGN, or were left untreated for the entirety of the experiment. This is one of 6 experiments with similar results.

Figure 6 shows another nitric oxide experiment comparing the same temperatures of 16°C, 19°, or 21°C. It was again found that a fever temperature of 19°C could, without any additional stimulation, cause an increase in nitric oxide production. However, in this experiment, the increased fever temperature of 21°C did not cause an increase in nitric oxide concentration in the untreated condition as it did in the previous experiment. It appears that at temperatures as high as 21°C, the cells respond very variably. This is likely because cell death often occurs at such high temperatures.

In this experiment, only stimulation with 50 ug/ml poly I:C was used because it had shown to best stimulate nitric oxide production in RTS11 cells in previous experiments. Stimulation by 50 ug/ml poly I:C caused an increase in nitric oxide production at normal body temperature of 16°C and at the increased fever temperature 21°C.

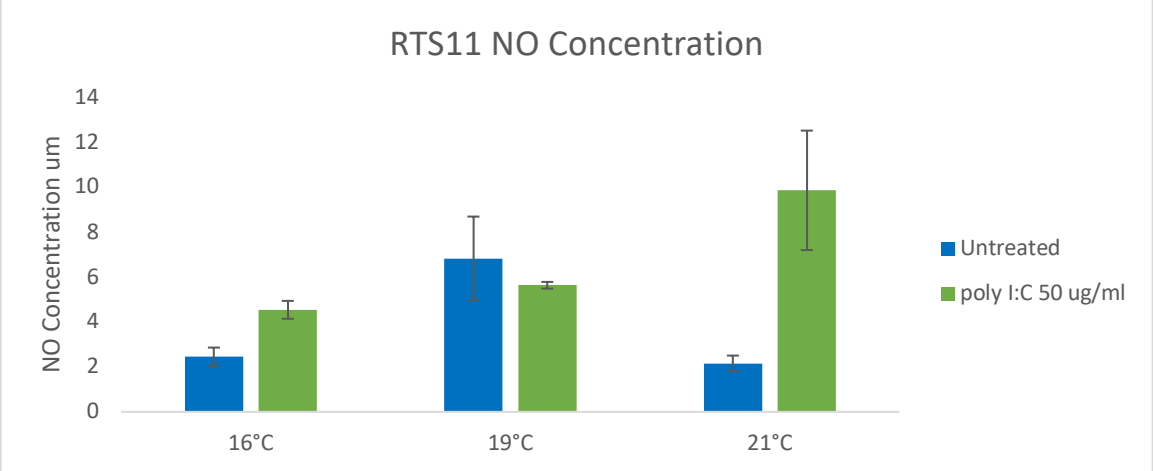


Figure 6. Impact of fever temperature and stimulation on nitric oxide production by RTS11 macrophages. Cells were incubated at 16°C, 19°, or 21°C for 48 hours. 100,00 cells were placed in each well. Cells were either stimulated immediately after plating with 50 ug/ml poly I:C or were left untreated for the entirety of the experiment. This is one of 6 experiments with similar results.

Many nitric oxide assay experiments were conducted prior to obtaining the above results. As RTS11 is new in the Durdik lab, many experiments had to be conducted in order to find the optimal growth and experimental conditions including FBS concentration in media, cell number, incubation time, stimulation time, and even the best incubators needed to be found or purchased. This data is included in the appendix.

Protein Synthesis

In order to analyze fluorescence data gathered from protein synthesis experiments, cells were processed through a flow cytometer. To analyze only single cells and not dust or cell debris, cells were gated based on parameters of cell size and cell complexity. Cells in the top right corner were excluded because they were likely doublets or clumps of cells rather than single cells. The bottom left corner was excluded because they are of a smaller size than intact cells. Therefore, the population of individual cells inside the gate represents single cells that were included in protein synthesis analysis.

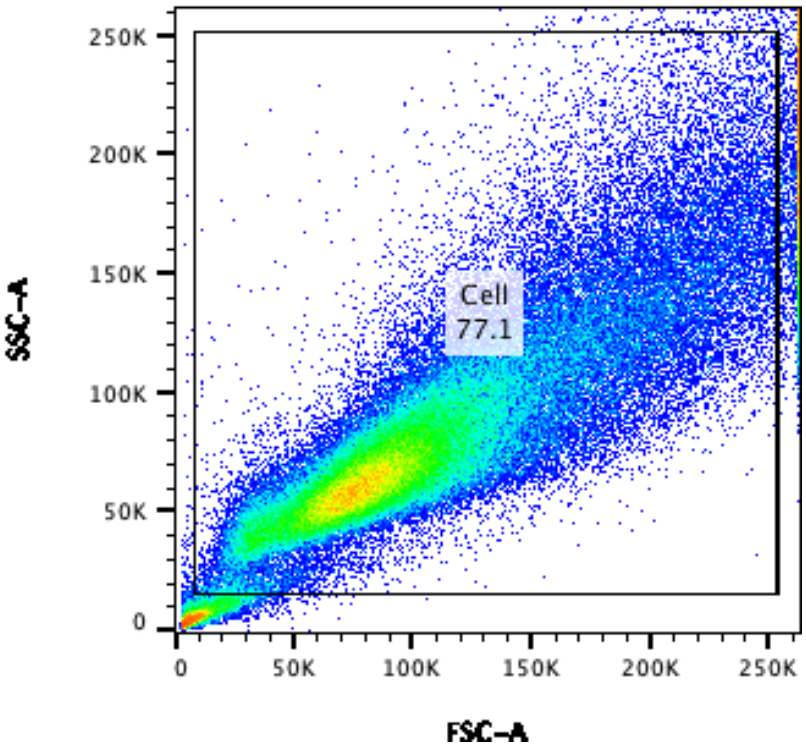


Figure 7. Depiction of the gating used to determine single cell count analyzing protein synthesis. This encompassed 77.1% of the data points produced.

Figure 8 compares the protein synthesis levels based on incubation temperature alone. 16°C serves as the control protein synthesis level as it is the normal body temperature of rainbow trout. 4°C typically serves as the negative control for protein synthesis, as other animals used in the lab such as mice and chicken do not synthesize any protein at this low temperature which would be shown in this assay by a uniform peak on the left side of the histogram. However, rainbow trout have a major low peak that then has a tail of higher signal to the right indicating that they may indeed make small amounts of protein even at 4°C. 19° and 20°C are the fever temperatures that were analyzed in this experiment. Both fever temperatures showed a similar increase in protein synthesis compared to the normal body temperature of 16°C. This is shown by the shift to the right on this histogram. The x-axis measures HPG by its fluorescence at a wavelength of 488 nm. Therefore, a shift to the right indicates an increase in incorporated HPG and therefore an increase in new protein synthesis. The y-axis measuring cell number was normalized to mode so that the protein synthesis levels could be accurately compared even if different numbers of cells were collected within the cell gate at the different temperatures.

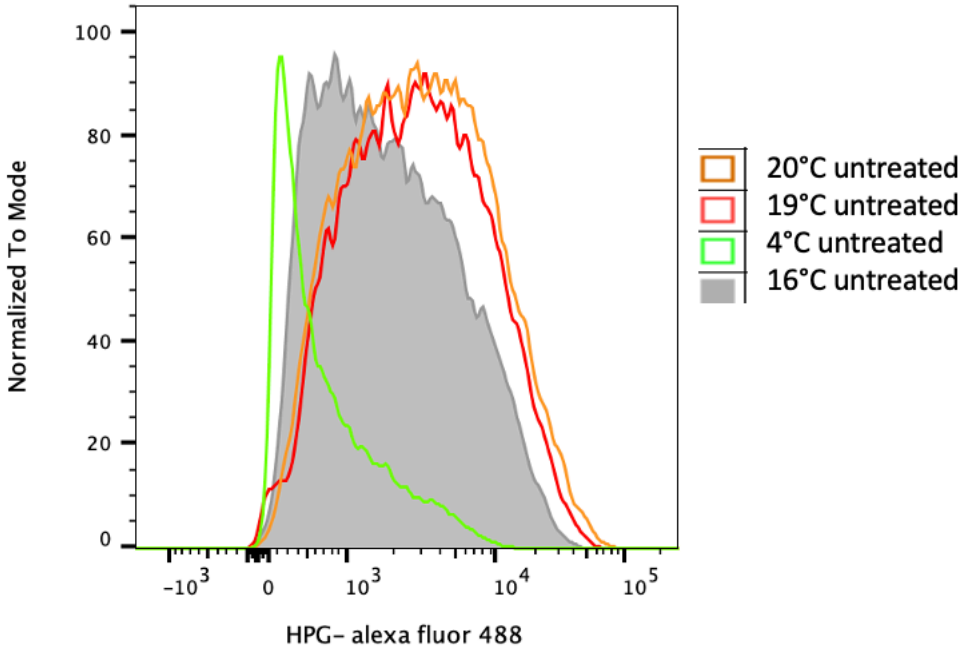


Figure 8. Protein synthesis levels of gated RTS11 macrophages. Cells were incubated at 4°C, 16°C, 19°, or 20°C for 4 hours. 1 million cells were placed in each well. 10,000 cells

were collected by the flow cytometer (before the gate). This is one of 5 experiments with similar results.

Figure 9 demonstrates the effect of stimulation by the viral mimic poly I:C on protein synthesis level. At each temperature, poly I:C caused an increase in global protein synthesis levels, indicated by many cells moving from low levels of incorporated HPG to higher levels.

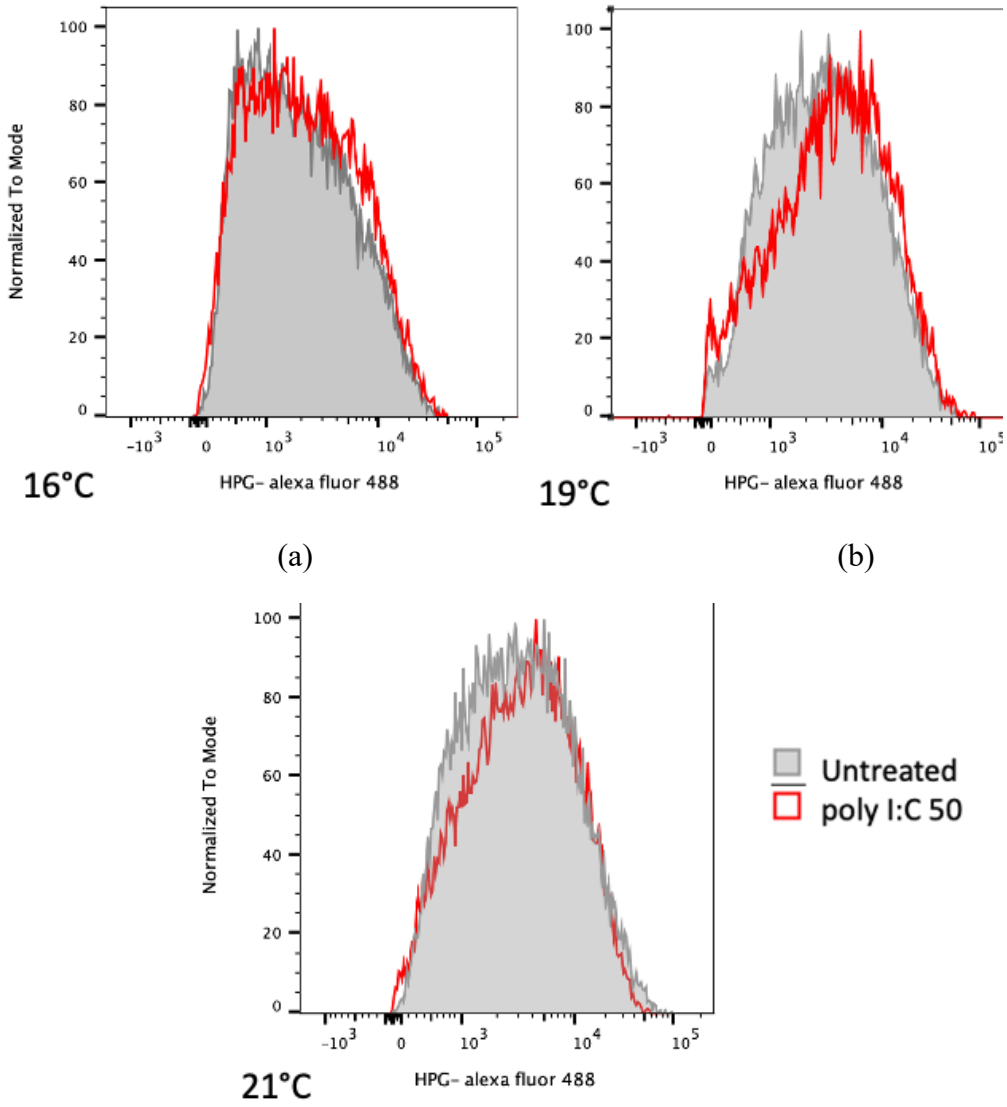
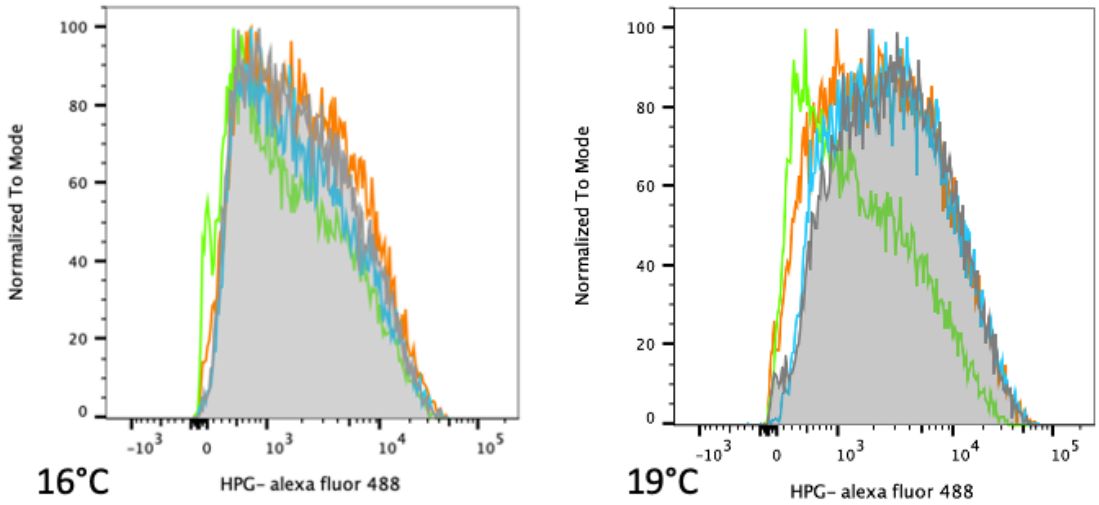


Figure 9. Comparison of protein synthesis levels in the untreated condition versus stimulated by viral mimic poly I:C. Cells were incubated at 16°C (a), 19° (b), or 20°C (c) both in the untreated condition and with stimulation by 50 ug/ml poly I:C for 4 hours. 1

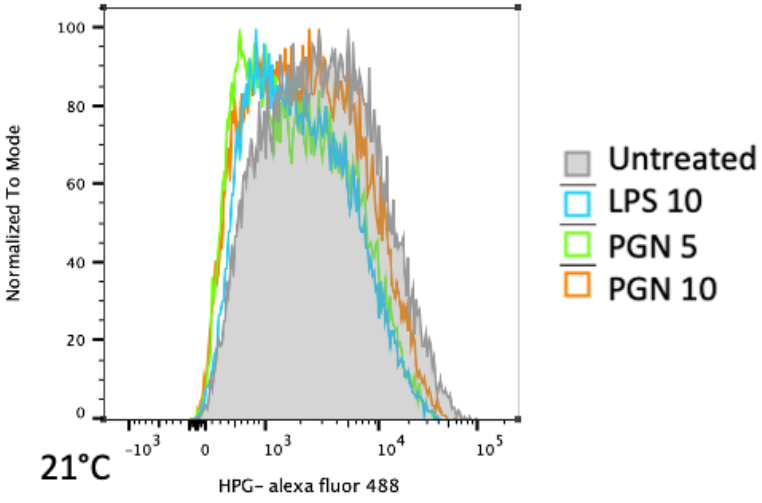
million cells were placed in each well. 10,000 cells were collected by the flow cytometer (before the gate). This is one of 5 experiments with similar results.

Figure 10 shows protein synthesis levels due to stimulation by two bacterial derived reagents LPS and PGN. At 16°C, LPS and the lowest concentration of PGN (5 ug/ml) caused a decrease in protein synthesis levels. However, a higher concentration of PGN did not affect the level of protein synthesis at 16°C. At 19°C, stimulation by 5 ug/ml of PGN caused a significant decrease in protein synthesis levels. However, again stimulation by LPS and PGN at 10 ug/ml did not change the level of protein synthesis. At 20°C, all stimulation conditions caused a decrease in protein synthesis. Consistent with the previous results, PGN at 10 ug/ml did not cause as much of a decrease.



(a)

(b)



(c)

Figure 10. Comparison of protein synthesis levels in the untreated condition versus stimulation by bacterial mimics LPS and PGN. Cells were incubated at 16°C (a), 19° (b), or 20°C (c) both in the untreated condition and with stimulation by 10 ug/ml LPS, 5 ug/ml PGN, or 10 ug/ml PGN for 4 hours. 1 million cells were placed in each well. This is one of 5 experiments with similar results.

Additional histograms with pairwise comparisons of the effects of temperature on each stimulation are included in the appendix.

Discussion

Nitric Oxide Assay

The major finding is that fever temperature alone can increase macrophage activity (without additional stimulation) as demonstrated in nitric oxide production. As shown in figures 5 and 6, nitric oxide production increased at an incubation temperature of 19°C. At temperatures above 19°C, results were highly variable. This is likely due to some cell death at these higher temperatures. The Durdik lab hypothesizes that the different populations we see under the microscope of RTS11, which are adherent and non-adherent macrophages, may act differently with the adherents representing a more mature population or perhaps simply one that is not busy dividing. The adherent cells come up off the plate to divide. It is also possible that these different populations could have different survival likelihoods at higher temperatures. Further research in the Durdik lab may analyze the differences within the adherent and non-adherent cell populations of RTS11 macrophages.

In accordance with this knowledge of adherent and non-adherent cell populations, it was hypothesized that giving the macrophages time to adhere before exposing them to stimulations would increase their immune response. However, it was found that this additional time for adherence had no effect on protein synthesis levels (appendix figure 1).

In addition to adding time before stimulation, FBS concentration in media, cell number, and incubation time were tested for optimal results. Initial experiments conducted with media with 30% FBS did not provide any data in which temperature and stimulation could be compared or analyzed. This result is in agreement with previous research done on RTS11 macrophages which says that high levels of FBS interfere with response to stimulation by LPS (Ganassin, et al). Therefore 0.1% FBS was used in media for experimentation. Like stimulation time, cell number did not greatly affect nitric oxide production severely, and so 100,000 cells per well was used in all subsequent nitric oxide assay experiments (appendix figures 2 and 3).

It was found that incubation time had effects on nitric oxide production. 48 hours appeared to be the best time for incubation, whereas 24 hours had been previously used in

the Durdik lab for mice and chicken NO assays (appendix figure 4). 72 and 96 hour incubation periods were also tested. There was decreased nitric oxide production at 72 hours, but increased production at 96 hours, especially at fever temperatures (appendix figure 5). It is therefore concluded that for incubation periods longer than 3 days at a high temperature, macrophages will demonstrate a large response. Consistent with our other results, this is not facilitated by stimulation, but by fever temperature alone.

Poly I:C, a viral mimic stimulated vigorous macrophage responses. At 16°C, poly I:C at a concentration of 50 ug/ml was able to consistently elicit a higher nitric oxide production (figures 5 and 6). At 19°C and 20°C the effect of poly I:C was more variable, causing increases in NO production in some experiments and not having a large effect in others. LPS and PGN did not appear to stimulate macrophage NO production greatly. It is possible that macrophage response to viral pathogens (poly I:C) and bacterial pathogens (LPS and PGN) differ. These results were also seen in the protein synthesis experiments discussed below.

Protein Synthesis

Protein synthesis experiments demonstrated that fever temperature alone can increase global protein synthesis (without stimulation). Figure 8 shows that fever temperatures of 19°C and 20°C increased protein synthesis levels compared to 16°C.

An interesting finding and consistent with our NO results was that the overall protein synthesis results showed higher responses to the viral mimic, poly I:C and lesser responses to the bacterial mimics, LPS and PGN. Poly I:C at a concentration of 50 ug/ml increased protein synthesis levels at all temperatures. Bacterial mimics were more variable, but either caused no change or a decrease in protein synthesis at all temperatures. We know that there are different pathways responsible for viral and bacterial responses but how these play into general metabolism as well as into specific activation pathways is new territory to understand. Further research in the Durdik lab will investigate these differences.

Conclusion

Unlike mammals and birds, ectothermic vertebrates such as fish are unable to physiologically increase their body temperatures very much. Therefore, they are limited to behavioral thermoregulation. Among ectothermic vertebrates, the preferred temperatures when the organism is ill varies greatly, however many of these organisms show elevations in preferred temperature when infected with a pathogen (Reynolds, et al).

Based on this research, a fever temperature of 19°C appears to be the preferred temperature for rainbow trout when fighting infections. In both nitric oxide and protein synthesis experiments, a fever temperature of 19°C consistently caused an increase in macrophage activity alone without any stimulation. As nitric oxide has an effect on rates of glycolysis in other species and therefore protein synthesis levels, it makes sense that strong correlation might be found in results from nitric oxide assay and protein synthesis experiments even in rainbow trout.

This research has found that fever temperature alone has the potential to greatly increase severe critical responses that initiate responses to infection in RTS11 macrophages. Further research will continue to work with viral and bacterial mimics as stimulations and look at the difference temperature makes in macrophage responses from rainbow trout. The establishment of optimal experimental conditions as discussed above will allow further research with both RTS11 cells and freshly isolated cells from head kidney and spleens of rainbow trout in the Durdik lab to be expedited.

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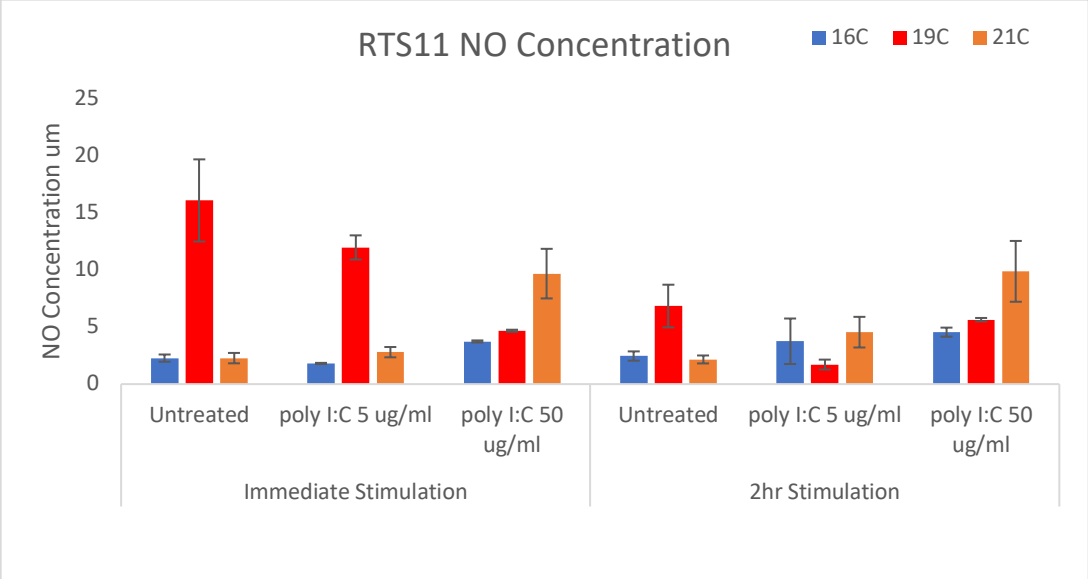
Appendix

Nitric Oxide Assay

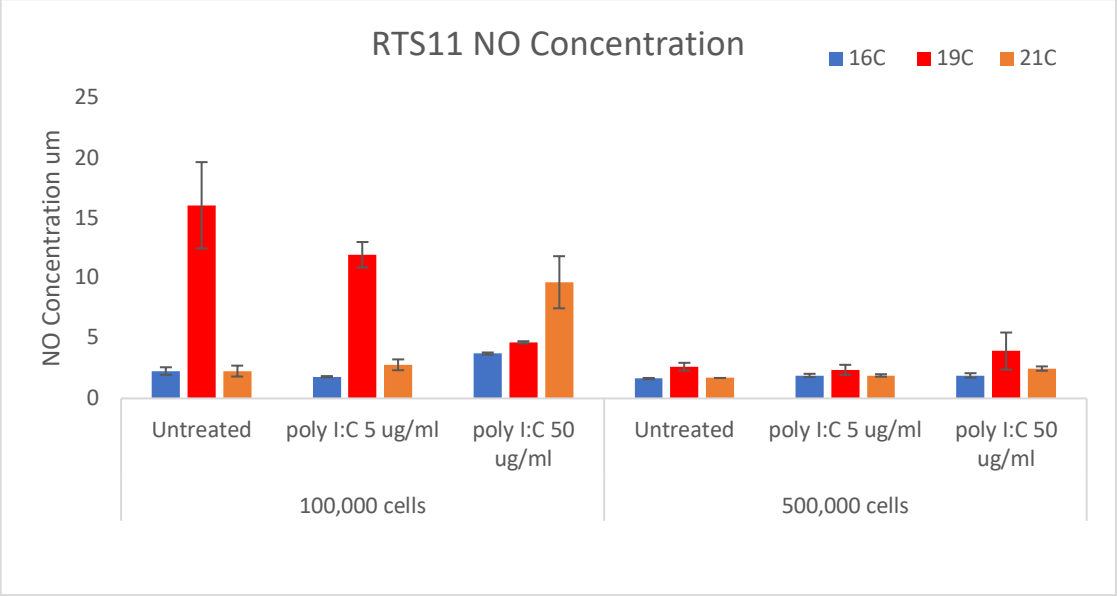
Appendix figure 1 shows an experiment in which one sample of cells was stimulated immediately after plating and another sample was stimulated after two hours on the plate. This was thought to allow the macrophages to adhere. However, there was either a decrease or no change in nitric oxide production due to allowing time for adherence prior to stimulation. Therefore, immediate stimulation was used in all subsequent nitric oxide assay experiments.



Appendix Figure 1. Impact of stimulation time on nitric oxide production by RTS11 macrophages. Cells were incubated at 16°C, 19°C, or 21°C for 48 hours. 100,00 cells were placed in each well. Cells were either stimulated immediately after plating or allowed to adhere to plates for 2 hours before stimulation. This is one of 2 experiments with similar results.

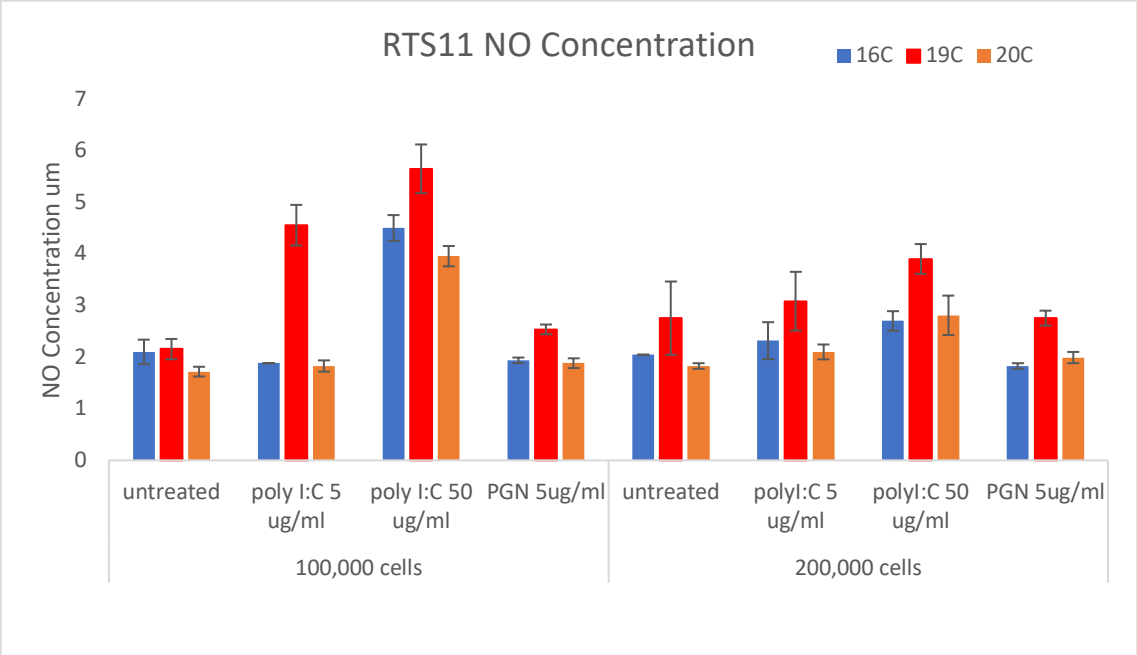
Appendix figure 2 shows an experiment in which one sample of cells was plated with 100,000 cells per well and another sample was plated with 500,000 cells per well. The samples with 500,000 cells per well had very little response at any temperature or condition. This is likely because cell death occurred as there were too many cells and not

enough nutrient media to keep cells alive. Thus, smaller numbers of cells per well were used in subsequent experiments.



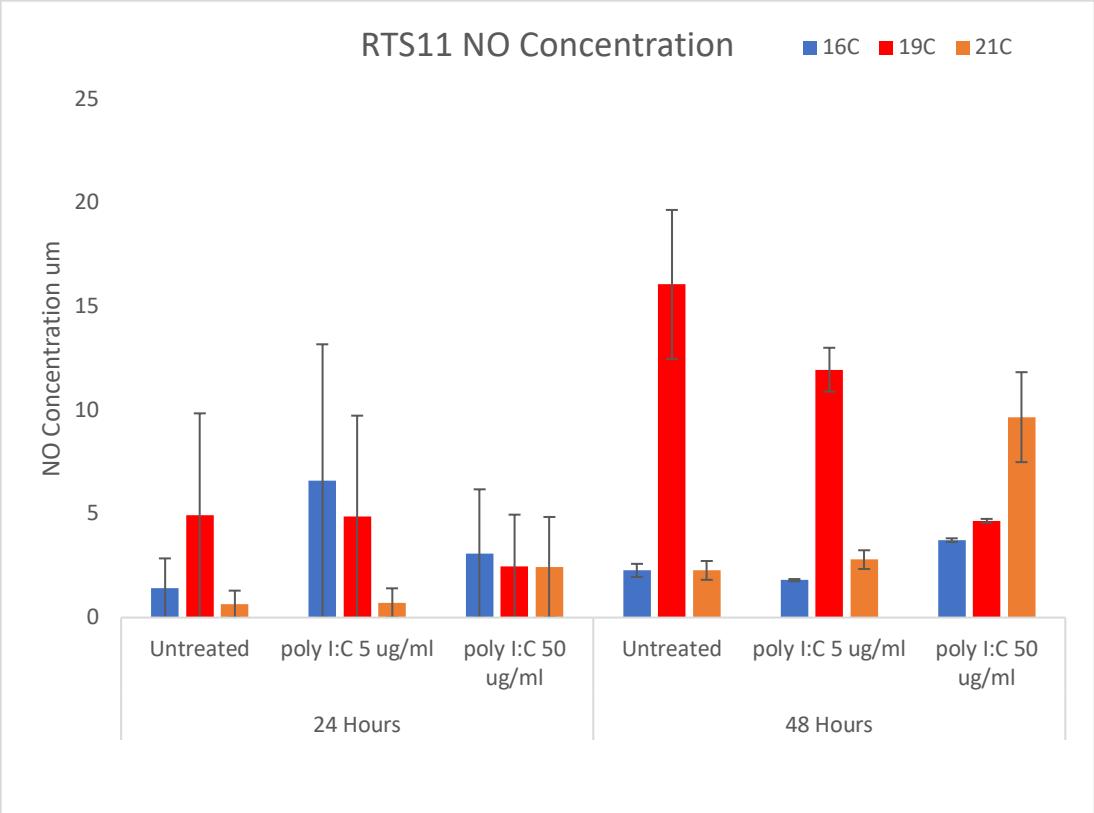
Appendix Figure 2. Impact of cell number on nitric oxide production by RTS11 macrophages. Cells were incubated at 16°C, 19°C, or 21°C for 48 hours. 100,000 or 500,000 cells were placed in each well and cells were stimulated immediately. This is one of 2 experiments with similar results.

Appendix figure 3 shows an experiment in which one sample of cells was plated with 100,000 cells per well and another sample was plated with 200,000 cells per well. The samples with 200,000 cells per well either did not show significant difference or showed a decrease in nitric oxide production when compared to the concentrations produced by the samples with 100,000 cells per well. Therefore, 100,000 cells per well was accepted as the optimal cell number for RTS11 NO experiments and was used in all subsequent nitric oxide assay experiments.



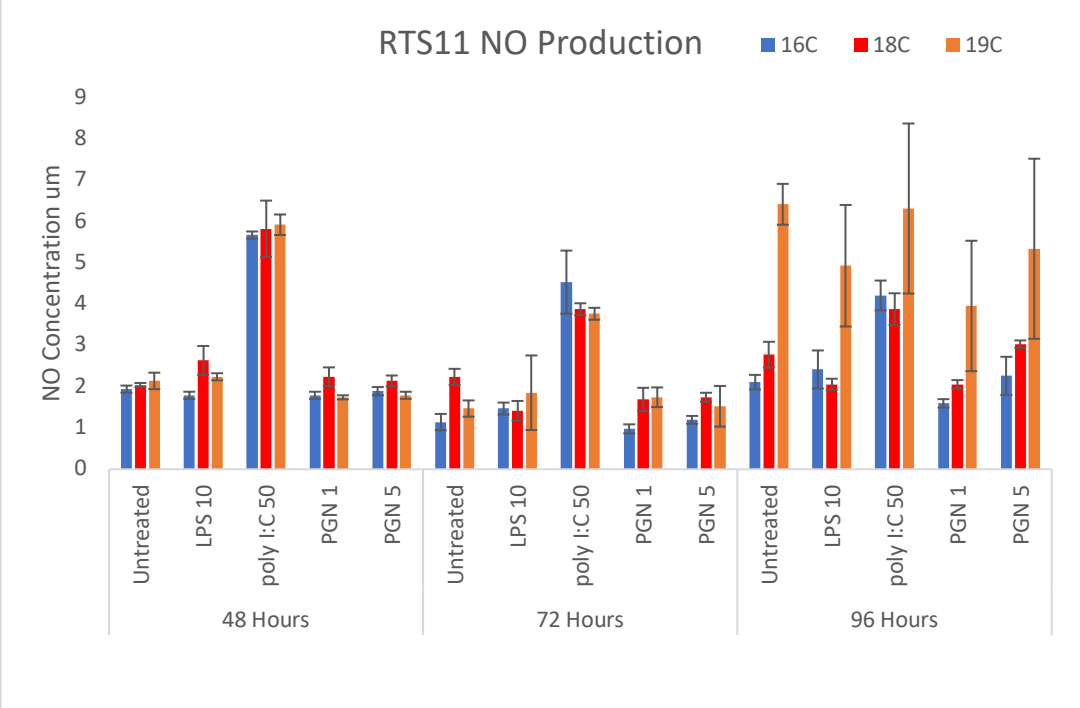
Appendix Figure 3. Impact of cell number on nitric oxide production by RTS11 macrophages. Cells were incubated at 16°C, 19°C, or 20°C for 48 hours. 100,000 or 200,000 cells were placed in each well and cells were stimulated immediately. This is one of 2 experiments with similar results.

Appendix figure 4 shows an experiment in which incubation times were 24 hours and 48 hours. At 24 hours, response was highly variable, shown by very large error bars. These error bars were far too large to draw any conclusions by comparison of temperatures and stimulations. Additionally, nitric oxide concentrations were higher at almost all conditions after 48 hours. Therefore, all subsequent nitric oxide assay experiments used an incubation time of 48 hours rather than 24 hours.



Appendix Figure 4. Impact of incubation time on nitric oxide production by RTS11 macrophages. Cells were incubated at 16°C, 19°C, or 21°C for 24 or 48 hours. 100,000 cells were placed in each well. Cells were stimulated immediately after plating. This is one of 4 experiments with similar results.

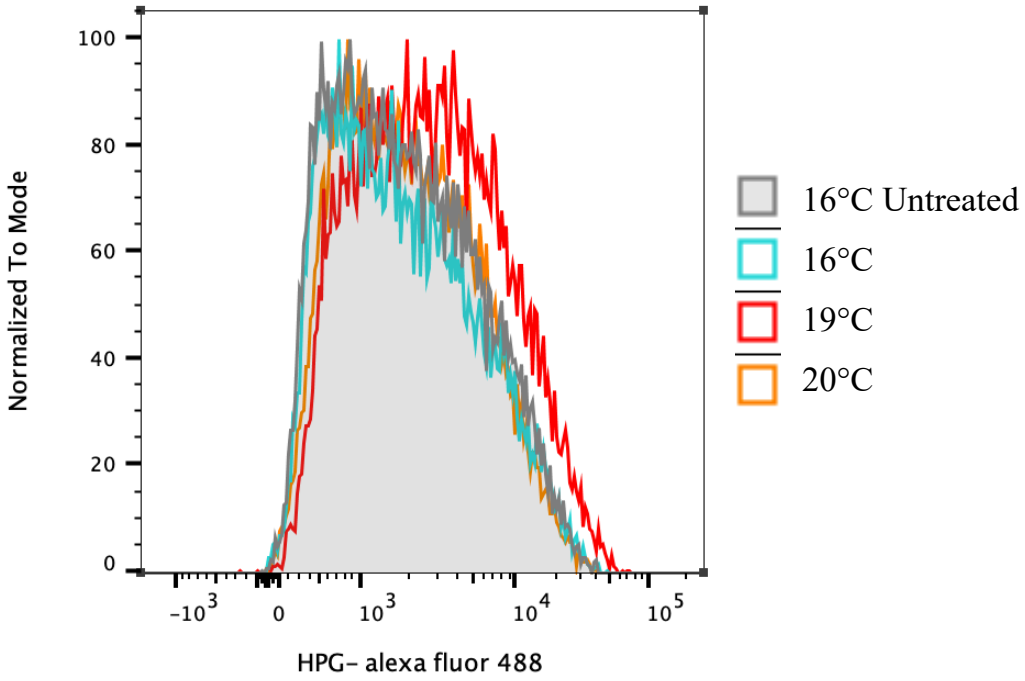
Appendix figure 5 shows an experiment in which cells were incubated at 48, 72, or 96 hours. Comparing 48 hours to 72 hours, there is either little change in the amount of nitric oxide produced or there is a slight decrease at 72 hours in some treatment conditions. At 96 hours however, there was an increase in nitric oxide production, especially at the fever temperature of 19°C. It appears that at incubation periods longer than three days, macrophages at fever temperatures will demonstrate a large, but variable response.



Appendix Figure 5. Impact of incubation time on nitric oxide production by RTS11 macrophages. Cells were incubated at 16°C, 18°C, or 19°C for 48, 72, or 96 hours. 100,000 cells were placed in each well. Cells were stimulated immediately after plating. This is one of 2 experiments with similar results.

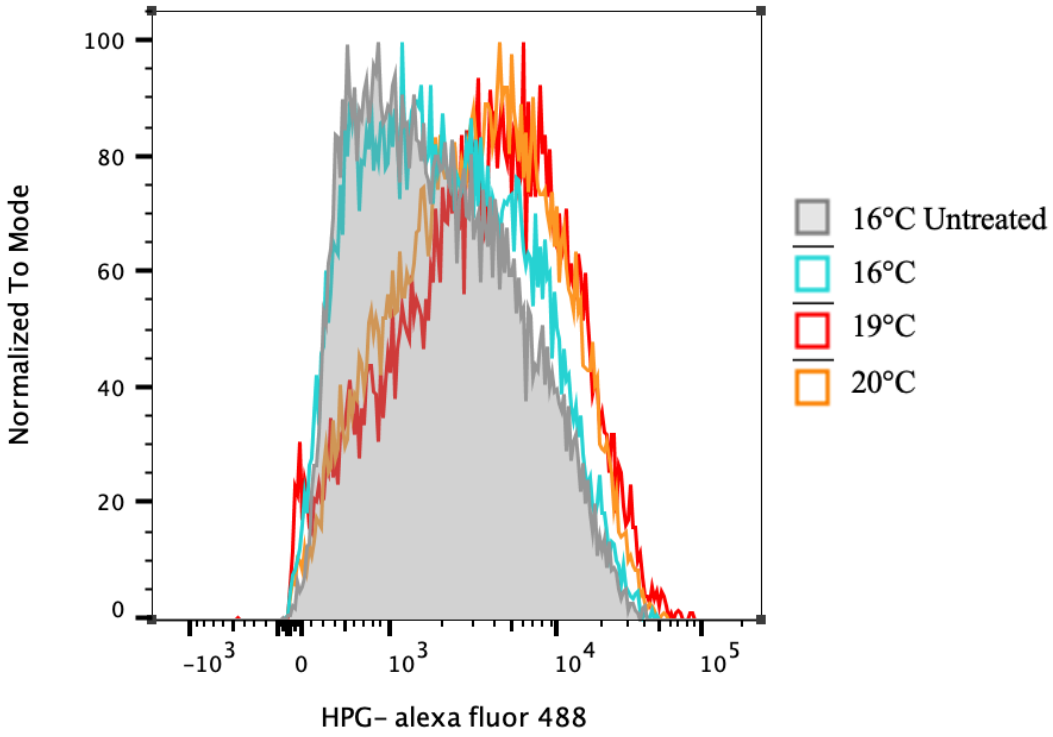
Protein Synthesis

Appendix figure 6 shows the effect of temperature on stimulation by LPS at a concentration of 10 ug/ml. There appears to be an increase in protein synthesis at 19°C compared with the control condition of 16°C untreated. However, figure 10(b) shows that this is due to temperature alone. At 20°C LPS causes no significant change in protein synthesis level, and at 16°C LPS causes a slight decrease in protein synthesis level from the production at 16°C alone.



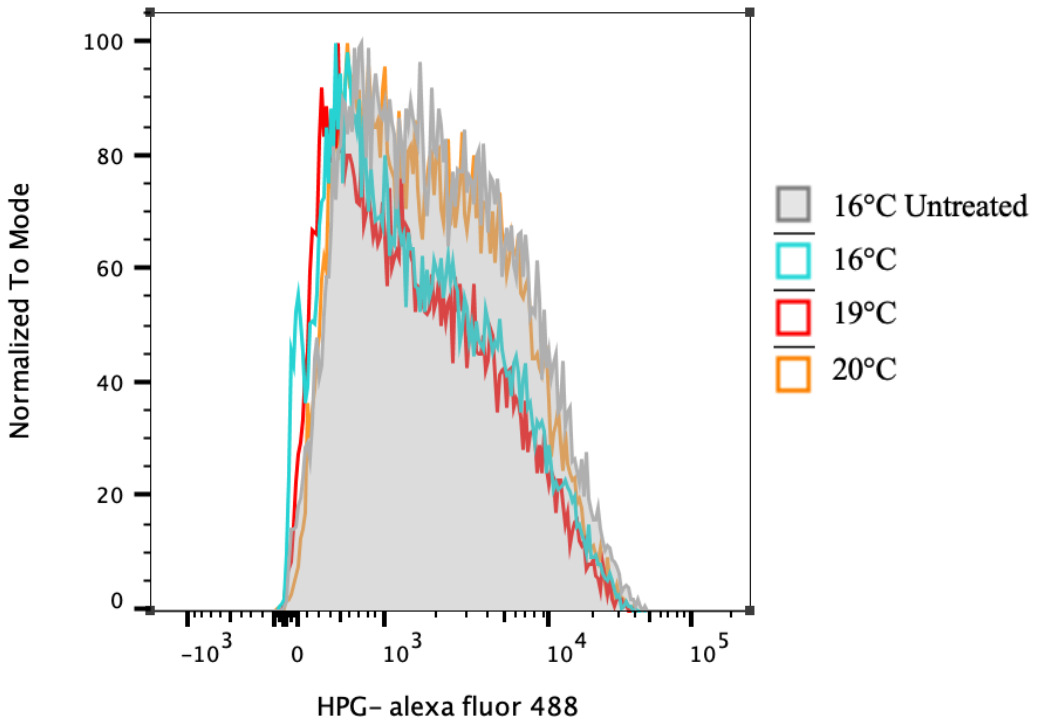
Appendix Figure 6. The effect of temperature on stimulation by LPS at a concentration of 10 ug/ml. Cells were incubated at 16°C, 19°, or 20°C for 4 hours. 1 million cells were placed in each well. 10,000 cells were collected by the flow cytometer (before the gate). This is one of 5 experiments with similar results.

Appendix figure 7 shows the effect of temperature on stimulation by poly I:C at a concentration of 50 ug/ml. Poly I:C caused an increase in protein synthesis at all temperatures (as shown in figure 9). However, the level of protein synthesis by 19°C and 20°C due to poly I:C stimulation appears to be equal.



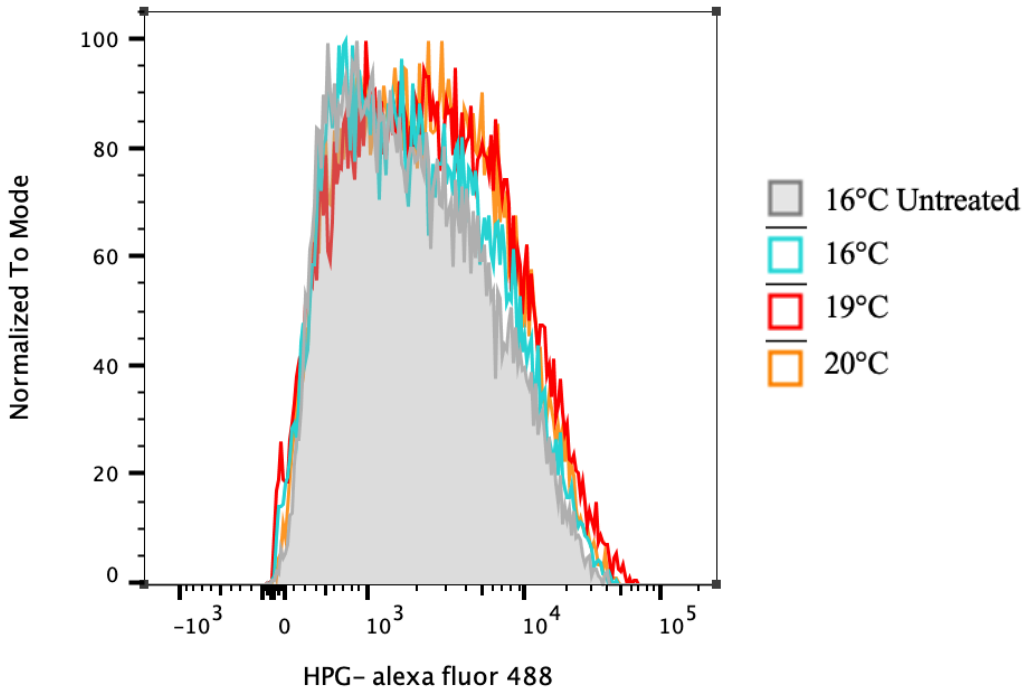
Appendix Figure 7. The effect of temperature on stimulation by poly I:C at a concentration of 50 ug/ml. Cells were incubated at 16°C, 19°, or 20°C for 4 hours. 1 million cells were placed in each well. 10,000 cells were collected by the flow cytometer (before the gate). This is one of 5 experiments with similar results.

Appendix figure 8 shows the effect of temperature on stimulation by PGN at a concentration of 5 ug/ml. At 16°C and 19°C, PGN at 5 ug/ml caused a decrease in protein synthesis levels from the background protein synthesis level. At 20°C protein synthesis was approximately equal to the control level. However, comparison with figure 10(c) shows that PGN 5 ug/ml causes a decrease in protein synthesis from the levels produced by a temperature of 20°C alone.



Appendix Figure 8. The effect of temperature on stimulation by PGN at a concentration of 5 ug/ml. Cells were incubated at 16°C, 19°, or 20°C for 4 hours. 1 million cells were placed in each well. 10,000 cells were collected by the flow cytometer (before the gate). This is one of 5 experiments with similar results.

Appendix figure 9 shows the effect of temperature on stimulation by PGN at a concentration of 10 ug/ml. At 16°C PGN at 10 ug/ml showed similar protein synthesis levels from the background protein synthesis level. At 19°C and 20°C protein synthesis was increased from the control level. However, comparison with figure 10(b) shows that PGN 10 ug/ml does not significantly change protein synthesis from the levels produced by the increased temperature of 19°C alone. Comparison with figure 10(c) shows that PGN 10 ug/ml causes a slight decrease in protein synthesis from the level produced by a temperature of 20°C alone.



Appendix Figure 9. The effect of temperature on stimulation by PGN at a concentration of 10 ug/ml. Cells were incubated at 16°C, 19°, or 20°C for 4 hours. 1 million cells were placed in each well. 10,000 cells were collected by the flow cytometer (before the gate). This is one of 5 experiments with similar results.